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(54) Title: A PROCESS FOR PROLIFERATION OF WHOLLY OR PARTIALLY DIFFERENTIATED BETA-CELLS

(57) Abstract

A process for proliferation of wholly or partially differentiated beta-cells comprises depositing islets or islet cultures from the pancreases of human or animal origin as monolayers on a solid substrate and keeping them in contact with a glucose-containing nutrient medium containing 1/2 - 7% human serum and 1 - 1000 ng growth hormone per ml nutrient medium. Cultivation of the beta-cells under such conditions provides continuous or long-term proliferation of the cells and a simultaneous strong increase in the insulin production.

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A process for proliferation of wholly or partially differentiated beta-cells

The present invention relates to a process of the type defined in the introductory portion of claim 1 for proliferation of wholly or partially differentiated beta-cells.

The type I diabetes mellitus disease is usually accompanied by progressive destruction of the insulin producing so-called beta-cells in the islets of Langerhans. Patients suffering from this disease are normally treated with daily injections of insulin recovered from the pancreas from animals, such as beef or pig, or produced by engineering. However, this mode of treatment is still incomplete, and complications at the advanced stages of the disease result in a high mortality rate among diabetics.

A considerably better regulation of the glucose content of the blood may be obtained by transplantation of tissue from pancrease or isolated islets of Langerhans, which is believed to reduce the risk of the mentioned complications considerably. However, for this treatment to be used on a larger scale, it is required that sufficient amounts of insulin producing tissue are provided. The number of available human pancrease organs is by and large the same as the number of kidney donors since the pancrease organs are obtained from the same deceased persons. Another source is pancrease from fetal tissue from abortions, but this source, too, is limited. Therefore, the ability of proliferating the insulin producing cells by cell cultivation is very important (ref. 1). It is possible to preserve functional islets of Langerhans, isolated from human pancreas, for months under ordinary

tissue culture conditions (see ref. 2 and ref. 3). However, these known processes involve no increase in the number of beta-cells in cultures of human islets of Langerhans from adults.

Even with rat islets of Langerhans, only limited beta-cell division has been observed, which has led to the general assumption that the postnatal division capacity of the beta-cell is poor, while some neoformation and/or division of beta-cells in vitro seems to occur in the embryonic and very late fetal state (see ref. 4). Only few cases of stimulation of beta-cell division in vitro have been reported, and it has generally been believed that glucose is the most important factor in cell division both in vivo and in vitro (ref. 4). However, experiments with mice and rats have demonstrated that growth hormone and related hormones, such as prolactin and placental lactogen, may exert a direct stimulating effect on the Langerhans's islets DNA synthesis and insulin production in vitro (ref. 5). However, this effect diminished as the cultivation period progressed, just as the islet growth in vivo decreases with the age of the animal.

The conventional method of cultivating cells is performed by depositing the cells on the surface of a solid substrate, e.g. of a plastics material. Such deposit is promoted by the presence of serum, e.g. 10% fetal calf serum, which is therefore widely used in the cultivation of insulin producing cells (ref. 6). Under these conditions, no effect of growth hormone was observed, either on the DNA synthesis or the insulin production (ref. 7). Increased insulin production is described (ref. 8), and an increased DNA synthesis without an increase in insulin secretion is likewise described (ref. 9 and ref. 10). While considerable, but time-limited stimulation of both insulin production and DNA synthesis has been demonstrated in the

intact islets (ref. 5), this has so far not been demonstrated in monolayer cultures.

The object of the present process is to provide an efficient and rational method of proliferating wholly or partially differentiated beta-cells in large amounts.

Another object of the invention is to proliferate cells which produce insulin in considerable amounts and which are useful for implantation in humans.

A further object of the invention is to produce human insulin.

The process of the invention, which is characterized by the features stated in the characterizing portion of claim 1, is based on the surprising finding that wholly or partially differentiated beta-cells develop strongly with formation of monolayers by cultivation on a solid substrate and in contact with a nutrient medium containing both serum and growth hormone in the stated concentration ranges, and when the nutrient medium is repeatedly exchanged during a cultivation period of preferably several weeks.

The nutrient medium used in the proliferation of the cells may expediently have a relatively high content of glucose, such as 1/2 - 10 g/l, preferably 1.5 - 5 g/l, with 2 g/l as the optimum value. The content of human serum is expediently 1/2 - 7%, preferably 1/2 - 3%, with about 2% as the optimum value. Growth hormone of animal or human origin may be replaced by hormones having similar properties, such as prolactin or placental lactogen.

The preferred concentration of growth hormone or the hormone having similar properties in the culture medium

is 10 to 1000 ng/ml, such as about 100 ng/ml. A considerable effect may also be obtained, however, at lower concentrations, such as down to about 1 ng/ml. Larger amounts of growth hormone may also be used, such as up to 1000 ng/ml culture medium or more, but, usually, no considerably improved cell formation is obtained at higher concentrations than 200 ng/ml.

The process of the invention permits proliferation of insulin producing cells of any type, also in the form of host cells into which one or more other gens which are to be expressed in animal or human cells have been introduced. Examples of this are cells in which DNA fragments which code for Factor VIII, growth hormone or interferon have been introduced.

The process of the invention will be illustrated more fully below by means of some working examples.

EXAMPLE 1

Pancrease from 15 three days old rats is excised, treated with collagenase, and the islets are isolated as described in ref. 5. 1000 islets are placed in culture medium RPMI 1640 containing 10% serum from newborn calves, distributed with 100 islets in 5 ml medium in Petri dishes. The dishes stand at 37 °C for 2 days as described in ref. 5. Then the islets are treated with a mixture of EGTA, DNase and trypsin and are aspirated until a cell suspension has been obtained, as described in ref. 11. The cells are suspended in medium RPMI 1640, and 10^5 cells in 5 ml medium are placed in cell cultivation dishes, as described in ref. 9. The dishes stand for 2 days at 37 °C, and the medium is then changed to RPMI 1640 admixed with 2% normal human serum and 100 ng/ml human growth hormone (Nanormon, Nordisk Gentofte, Denmark). The dishes stand at 37 °C,

and the medium is changed once a week. Insulin is measured by radioimmunoassay, as described in ref. 5.

The cells proliferate to colonies, and after 5 months the colonies have spread so that they cover almost the entire dish. They are treated with a mixture of EGTA, DNase and trypsin, and the resulting cell suspension is placed in new dishes with the media RPMI 1640 admixed with 2% normal human serum and 100 ng/ml human growth hormone. Again the cells proliferate to colonies and spread in the dish. The insulin production increases again gradually.

The results are shown in figure 1 in which curve I refers to the experiment performed in this example. Curve II refers to a comparative example performed in the same manner, except that no growth hormone was added.

EXAMPLE 2

A cell suspension is produced as described in example 1. 10^5 cells suspended in 100 µl serum-free medium RPMI 1640 are placed in cell cultivation dishes, and immediately following this are added 5 ml medium RPMI 1640 containing 0.5%, 1%, 2%, 5% and 10% human serum, respectively, as well as 1 µg/ml human growth hormone. The dishes stand at 37 °C, and the medium is changed once a week. The measured insulin amount, released to the medium, is depicted in figure 2 as a function of serum concentration and cultivation time. Maximum insulin production is obtained after 3 weeks cultivation, as shown, at a serum concentration of 2%.

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Patent Claims

1. A process for proliferation of wholly or partially differentiated beta-cells, comprising suspending islets or islets cultures isolated from human or animal pancrease in a culture medium and contacting them with a solid substrate until the islets or islet cultures have been deposited on the substrate, and cultivating the islets or islet cultures deposited on the solid substrate in a culture medium under conditions where the beta-cells form monolayers during their growth, c h a r a c t e r i z e d by culturing in a nutrient medium containing growth hormone or a hormone having similar properties in an amount of 1 - 1000 ng/ml and serum in an amount of 1/2 - 7%, and exchanging the culture medium repeatedly during a cultivation period of several days, preferably several weeks.

2. A process according to claim 1, c h a r a c t e r - i z e d by performing the cultivation for a period of at least 2 weeks, and exchanging the culture medium several times, such as once a week.

3. A process according to claims 1 or 2, c h a r a c - t e r i z e d in that the culture medium used for the cultivation of the cells deposited on the solid substrate contains glucose in an amount of 1/2 - 10 g per litre, preferably 1.5 - 5 g per litre, serum in an amount of 1/2 - 7%, preferably 1/2 - 3%, as well as growth hormone or a hormone having similar properties in an amount of 10 - 1000 ng/ml.

4. A process according to any of claims 1-3, c h a r a c t e r i z e d by using human serum and human growth hormone.

5. A process according to any of claims 1-4, characterized in that the cells used in the proliferation originate from pancrease from fetal tissue.

Fig. 1

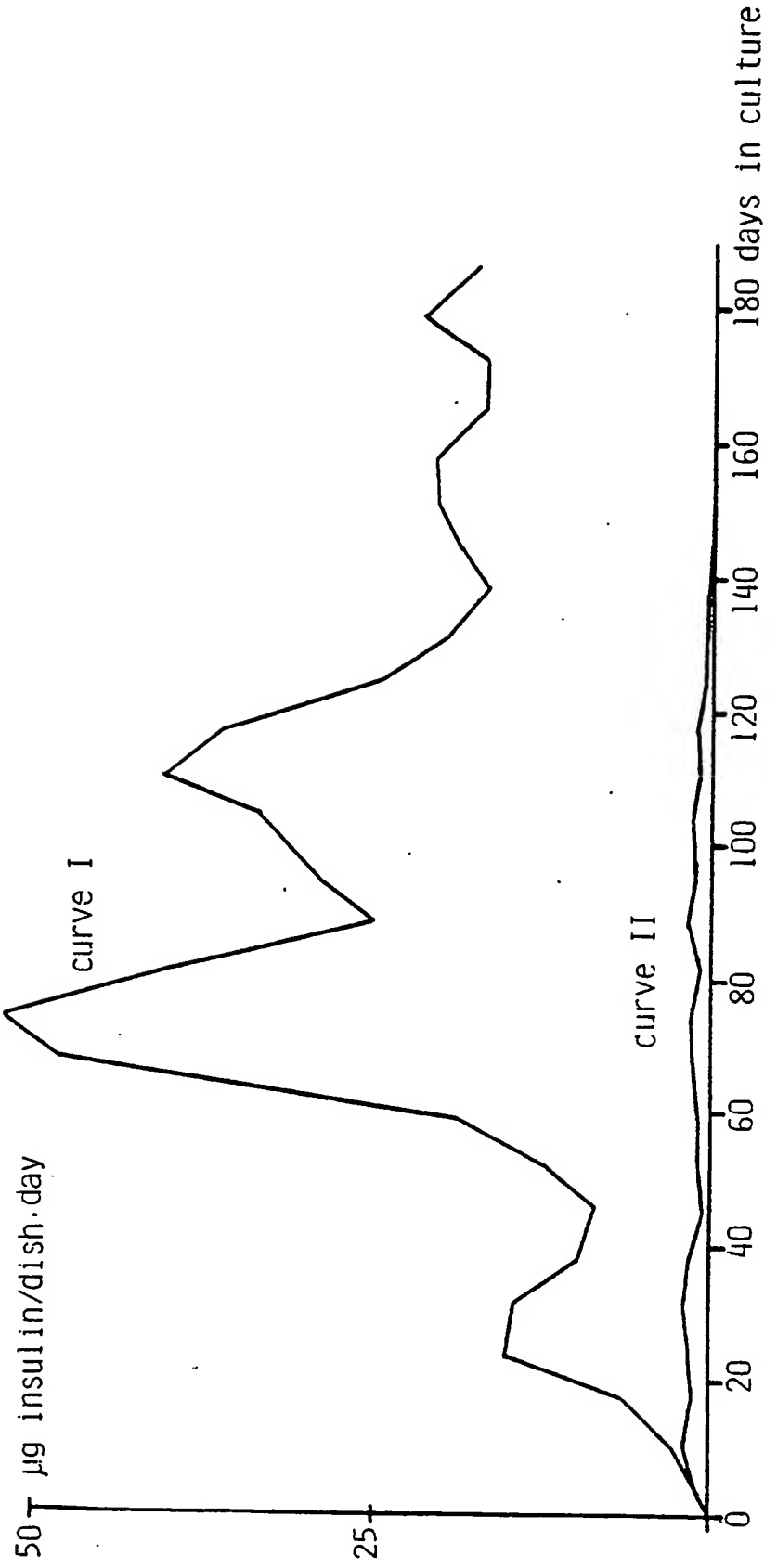
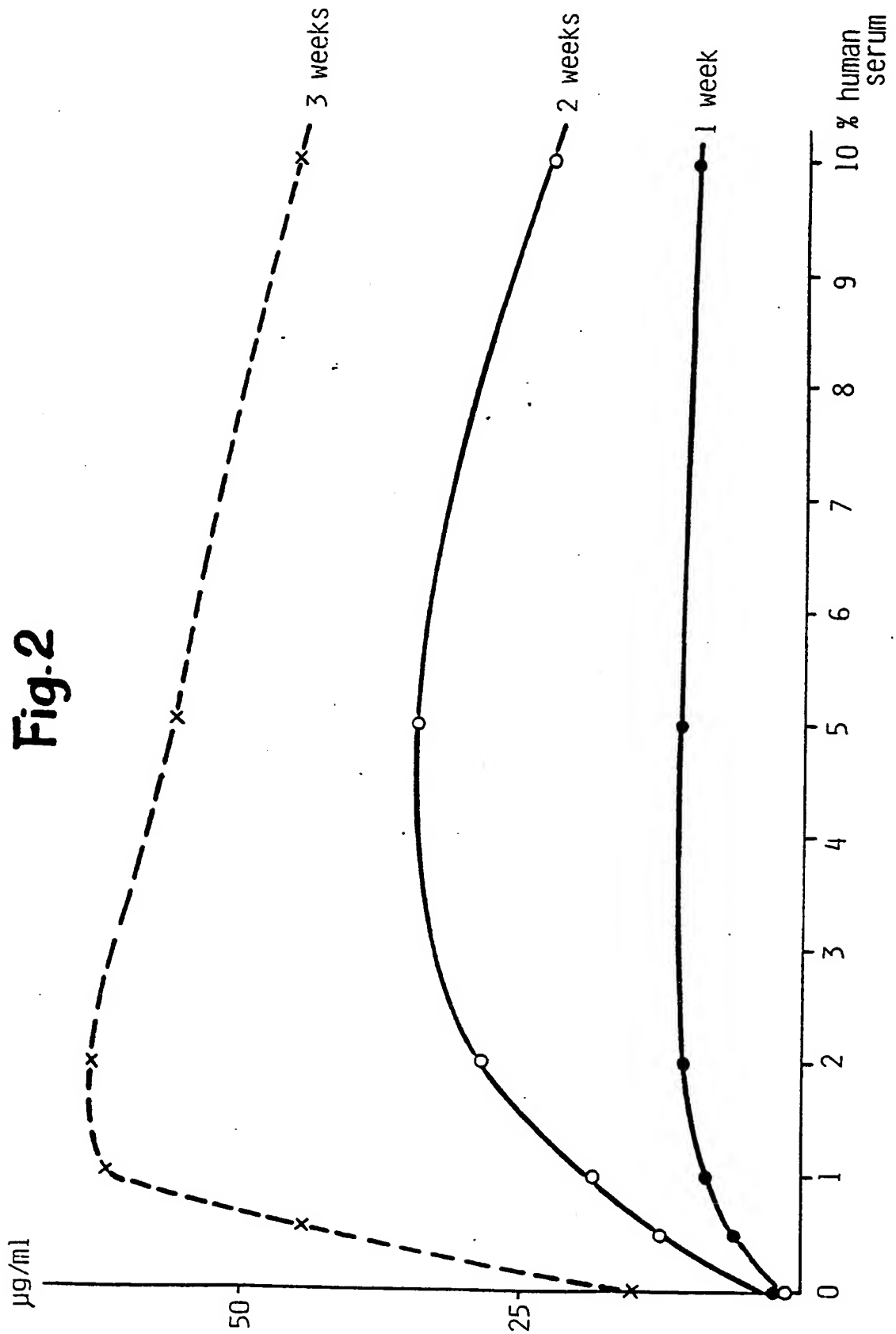

µg insulin/dish.day
50
25
0
curve I
curve II
0
20
40
60
80
100
120
140
160
180 days in culture

Fig.2



INTERNATIONAL SEARCH REPORT

International Application No PCT/DK85/00083

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

C 12 N 5/00 // C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
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US Cl	435:68, 71, 240

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE, NO, DK, FI Classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Growth of Cells in Hormonally Defined Media, Cold Spring Harbor Conf. on Cell Proliferation Vol 9, published 1982 (Ed. Sato G.H. et al) by Cold Spring Harbor Laboratories, see pages 501-506, especially p 502. "Cell Culture".	1-5
Y	Diabetes Vol 32 p 307-12, published 1983 (Rabinovitch A. et al) "Growth Hormone Stimulates Islet B-Cell Replication in Neonatal Ray Paneratic Monolayer Cultures." see especially tabel 2 and fig. 1B.	1-5
Y	Dissertation Abstracts Int. B Vol. 42 (1982), no 12, part 1, 4646.	1, 2
Y	Chemical Abstracts Vol 88 (1978), abstract 83813h, J.Endocrinol. 1977, 75(3), p 49-50.	1
A	US, A, 4 225 671 (BATTELLE-INSTITUTE E.V.) 30 September 1980, see particularly col 5, line 35.	1-3

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	DE, A, 2 757 169 (HOECHST AG) 5 July 1979	1
A	GB, A, 2 083 826 (KABUSHIKI KAISHI HAYASHIBARA SEIBUTSU) 31 March 1982	1